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(54) Title: COMPOSITION FOR PREVENTION AND TREATMENT OF PERIODONTAL DISEASES			
			
(57) Abstract			
<p>The present invention relates to a composition for the prevention and treatment of periodontal disease. More specifically, the present invention relates to a composition for the prevention and treatment of periodontal disease comprising an extract of Zizyphi fructus and an extract of Magnoliae cortex. When the extract of Zizyphi fructus and the extract of Magnoliae cortex are combined in the weight ratio ranging from 1:6 to 1:12 and, preferably from 1:8 to 1:10, they together show a synergistic effect in comparison to the single use of any one of them, alone, and further exhibit an excellent effect for treatment of periodontal diseases in comparison with the agents disclosed in the prior art for prevention and treatment of periodontal diseases.</p>			

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# COMPOSITION FOR PREVENTION AND TREATMENT OF PERIODONTAL DISEASES

## TECHNICAL FIELD

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The present invention relates to a composition for the prevention and treatment of periodontal diseases. More specifically, the present invention relates to a composition for the prevention and treatment of periodontal diseases comprising an extract of *Zizyphi fructus* and an  
10 extract of *Magnoliae cortex*. When the extracts of *Zizyphi fructus* and *Magnoliae cortex* are combined so that the ratio of the weight of the former to the weight of the latter ranges from 1:6 to 1:12, and more particularly from 1:8 to 1:10, their combination exhibits a synergistic  
15 effect in treating periodontal disease that is superior to the sum of their individual effects. Moreover, the combination is excellent for preventing and treating periodontal disease compared to agents disclosed in the prior art.

20

## BACKGROUND ART

Oral diseases are generally classified as dental caries and periodontal diseases. A periodontal disease is an infectious oral disease which most frequently occurs in adult man, and results in the loss of  
25 teeth due to gingival bleeding and swelling, formation of periodontal inflammation and disruption of alveolar bone, and the like. According to the currently accepted mechanism for the occurrence of periodontal disease, plaque is mechanically accumulated in periodontal pocket and then serves as a habitat for microorganisms. These microorganisms  
30 gradually change from aerobic gram-positive bacteria to anaerobic gram-negative bacteria as they penetrate deep into the periodontal pocket. Fully grown anaerobic gram-negative bacteria produce toxins and other substances which directly disrupt the periodontal tissues and trigger the immunologic system. The stimulated immunological system induces the  
35 disruption of alveolar bone and the inflammation of periodontal tissues

through various mechanisms. The causative microorganisms of periodontal disease are *Porphyromonas gingivalis*, *Prevotella intermedia*, *Actinobacillus actinomycetemcomitans*, etc., which are all gram-negative anaerobic bacteria normally present in the oral cavity, in contrast to the  
5 causative organisms of dental caries. In addition, periodontal diseases generally occur in adult man because periodontal tissues are weakened with age.

Therefore, in order to prevent or treat the periodontal diseases, it  
10 is of course preferable to eliminate all preconditions and causes of the diseases, that is, (1) to restore the weakened or disrupted periodontal tissues to their original state, (2) to remove the inflammation as the toxic production from causative microorganisms, and (3) to inhibit the reproduction of these microorganisms.

15

Studies of the prevention and treatment of periodontal disease have been conducted since the 1920's and, particularly in Europe and North America, have focussed on the antibacterial action of antibiotics and chemotherapeutic agents (for example, chlorhexidine, cetylpyridinium  
20 chloride) to remove plaque.

However, the use of such antibiotics and chemotherapeutic agents has undesirable side effects such as ulceration of oral mucous membranes, induction of desquamative gingivitis, colouring, etc.

25

In addition to the antibiotics and chemotherapeutic agents, an enzyme preparation that decomposes plaque stroma, and a fluorine preparation that inhibits the adsorption of bacteria onto the surfaces of teeth have also been disclosed for preventing periodontal disease. These,  
30 however, have seen limited use because the enzyme preparation has been shown to have harmful side effects and is also difficult to apply and the fluorine preparation exhibits only a weak antibacterial effect.

To overcome these disadvantages of antibiotics and other  
35 synthetic medicinal products, many attempts have made to develop a

medicinal drug for treatment of periodontal disease using extracts of medicinal herbs. As a result of this work, Myrrha has been shown to be effective against gingivitis and thrush, and sanggenon C, the active component of the extract of *Mori radice cortex*, has been shown to  
5 inhibit the metabolism of *Streptococcus mutans* and thus to obstruct bacterial adhesion, which is the first step in plaque formation. However, Myrrha exhibits only a weak antibacterial effect and the extract of *Mori radice cortex* considerably inhibits the growth of gingival fibroblast. Although the extract of *Sanguinaria* has been experimentally shown to be  
10 a potent plaque inhibitor and antibacterial agent, it has not demonstrated these effects in clinical studies.

In addition, the extract of *Zea mays L.* which is marketed in Korea under the trade names Insadol (Dongkuk Pharmaceutical Co., Ltd.)  
15 and Dentol (Lucky Co., Ltd.) has been used for inhibiting the inflammation of periodontal tissues.

Although such prior agents for the treatment of periodontal disease using medicinal herbs have the advantage that they have no  
20 harmful side effects, their shortcomings are that they have neither an regenerative effect on damaged cells of periodontal tissue nor an antibacterial effect on causative organisms of periodontal disease.

Thus, the present inventors have intensively studied various  
25 medicinal herbs in order to develop a therapeutic agent which overcomes the above mentioned disadvantages of the prior art agents for treatment of periodontal disease using medicinal herbs. As a result, the present inventors have found that the extract of *Magnoliae cortex* shows a good anti-inflammatory effect and a potent antibacterial effect on the causative  
30 organisms of periodontal disease, without the harmful side effects exhibited by the prior art antibiotics (see, Korean Laid-open Patent Publication No. 94-6594) and that the extract of *Ginkgo folium* has a regenerative effect on periodontal tissues (see, Korean Laid-open Patent Publication No. 94-13606). The present inventors have also shown that  
35 the combination of the extract of *Magnoliae cortex* and the extract of

Ginkgo folium is effective against periodontal diseases and, indeed, have filed a patent application relating to a composition for treatment of periodontal diseases comprising just such a combination (see, Korean Patent Application No. 94-14117). As a result of still further study, the present inventors have also identified that the extract of Zizyphi fructus is effective for regenerating periodontal tissues, and have filed a patent application relating to an agent for treatment of periodontal disease comprising the extract of Zizyphi fructus (see, Korean Patent application No. 95-14541).

Although the extract of Magnoliae cortex displays good anti-inflammatory and antibacterial activities, it has the disadvantage of lowering the activity of periodontal tissue cells as its dosage increases. Meanwhile, the extracts of Zizyphi fructus and Ginkgo folium together exhibit a good tissue regenerating effect but also have a weak antibacterial effect. Therefore, they are disadvantageous in view of the fact that they exhibit the effect through only some ways among various mechanisms involved in the prevention and treatment of periodontal diseases.

On the basis of the result of previous studies mentioned above, the present inventors have examined the efficacy of various combinations of the extracts of Magnoliae cortex and Zizyphi fructus for preventing and treating periodontal diseases. As a result, we have discovered that the combination of these two extracts at certain weight ratios shows a unexpected synergistic effect for treating periodontal diseases (i.e. excellent tissue regenerating, antibacterial and anti-inflammatory effects) and overcomes the disadvantage of the extract of Magnoliae cortex alone (i.e. inhibits the activity of periodontal tissue cells at high dosage) to provide a good tissue regenerating effect. Then, we have completed the present invention.

## DISCLOSURE OF INVENTION

The present invention relates to a composition comprising the

extract of *Zizyphi fructus* and the extract of *Magnoliae cortex* as its effective components which, at certain weight ratios of its active components, is broadly effective for preventing and treating periodontal diseases (i.e. exhibits (1) a regenerative effect on weakened or disrupted periodontal tissues, (2) an inhibitory effect on the inflammatory response which disrupts the periodontal tissues, and (3) an antibacterial effect on causative organisms of periodontal diseases) and which is far more effective than each of the extracts of *Zizyphi fructus* and *Magnoliae cortex* alone. In addition, the composition of the present invention has the additional advantage that it has no harmful side effects such as those exhibited by antibiotics of the prior art.

### BRIEF DESCRIPTION OF DRAWINGS

For a thorough understanding of the nature and objects of the invention, reference should be made to the following detailed description taken in connection with the accompanying drawings in which:

Figure 1 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the fifth group, which was used as the negative control group according to Experiment 4, 2 weeks after the defect operation. This photograph shows that at both sides of the defected portion having diameter of 5mm in the calvaria some new bone has formed in line but that the whole defected portion has filled with fibrous connective tissues.

Figure 2 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the third group (unsaponified extract of *Zea mays*, 0.1g/kg), which was used as the positive control group according to Experiment 4, 2 weeks after the defect operation. This photograph shows that most of the defected portion having diameter of 5mm in the calvaria has filled with fibrous connective tissues and that new bone and bone-like tissues have formed around the existing defected bone tissue.

Figure 3 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the fourth group (unsaponified extract of *Zea mays*, 0.5g/kg), which was used

as the positive control group according to Experiment 4, 2 weeks after the defect operation. This photograph shows that most of the defected portion having diameter of 5mm in the calvaria has filled with fibrous connective tissues and that new bone and bone-like tissues have formed within the inner bone-defected space but that the total unification has not occurred.

Figure 4 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the first group (present composition, 0.1g/kg), which was used as the test group according to Experiment 4, 2 weeks after the defect operation. This photograph shows that the defected portion having diameter of 5mm in the calvaria has filled with connective tissues and new bone and bone-like tissues have actively formed at the existing defected bone tissue but not fill all the bone-defected space, and that the total unification has not occurred.

Figure 5 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the second group (present composition, 0.5g/kg), which was used as the test group according to Experiment 4, 2 weeks after the defect operation. This photograph shows that the defected portion having diameter of 5mm in the calvaria has fully filled with newly formed bone and bone-like tissues, that some unification has occurred, and in the bone-like tissue active bone cells are found and new bone is greatly produced.

Figure 6 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the fifth group, which was used as the negative control group according to Experiment 4, 3 weeks after the defect operation. This photograph shows that the formation of new bone and bone-like tissues has initiated at both sides of defected portion having diameter of 5mm in the calvaria and has actively penetrated into the middle part, but the middle part of the bone-defected portion has still filled with fibrous connective tissues.

Figure 7 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the third group (unsaponified extract of Zea mays, 0.1g/kg), which was used as the positive control group according to Experiment 4, 3 weeks after



the defect operation. This photograph shows that the formation of new bone and bone-like tissues has begun at both sides of defected portion having diameter of 5mm in calvaria and has actively penetrated into the middle part, but the middle part has filled with fibrous connective tissues, and the type of newly formed bone and bone-like tissues is similar to that of Figure 6.

Figure 8 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the fourth group (unsaponified extract of Zea mays, 0.5g/kg), which was used as the positive control group according to Experiment 4, 3 weeks after the defect operation. This photograph shows that the formation of new bone and bone-like tissues has actively initiated at both sides of defected portion having diameter of 5mm in calvaria and attained up to near the middle part of bone-defected portion, but the middle part of the bone-defected portion has filled with some fibrous connective tissues, and the formation of new bone is greater than that in Figures 6 and 7.

Figure 9 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the first group (present composition, 0.1g/kg), which was used as the test group according to Experiment 4, 3 weeks after the defect operation. This photograph shows that the formation of new bone and bone-like tissues has actively progressed from both sides of the defected portion having diameter of 5mm in calvaria to fill all the bone-defected portion with bone-like tissue and the bone-like tissues are rapidly replaced with new bone.

Figure 10 is a photograph ( $\times 40$ ) of the calvaria of a mouse of the second group (present composition, 0.5g/kg), which was used as the test group according to Experiment 4, 3 weeks after the defect operation. This photograph shows that the formation of new bone has actively progressed from both sides of the defected portion having diameter of 5mm in calvaria to fill all the bone-defected portion with new bone and the unification is perfectly occurred.

## BEST MODE FOR CARRYING OUT THE INVENTION

In the composition of the present invention, the extract of Magnoliae cortex is best obtained by extracting Magnoliae cortex with an alcohol, preferably with a lower alkanol such as ethanol. It is also  
5 preferable to use an extract of Magnoliae cortex containing at least 5.0% of magnolol and at least 1.0% of honokiol as its major ingredients. In addition, the extract of Zizyphi fructus is best obtained by extracting Zizyphi fructus with an alcohol, preferably with a lower alkanol such as  
10 ethanol, in a water bath and then concentrating the extract under reduced pressure.

The composition comprising the extracts of Zizyphi fructus and Magnoliae cortex as its active components according to the present  
15 invention exhibits a surprising synergistic effect for treating periodontal diseases when these extracts are present in specific proportions. Specifically, as demonstrated by the experiments detailed below, a synergistic effect is evident when the ratio of the weight of the extract of Zizyphi fructus to the weight of the extract of Magnoliae cortex  
20 ranges from 1:6 to 1:12, and preferably from 1:8 to 1:10.

Therefore, to maximize the synergistic therapeutic effect on periodontal diseases the composition of the present invention uses the extract of Zizyphi fructus and the extract of Magnoliae cortex in the  
25 mixing ratio ranging from 1:6 to 1:12, particularly preferably in the mixing ratio ranging from 1:8 to 1:10, on the basis of weight.

Although the composition of the present invention comprising the extracts of Zizyphi fructus and Magnoliae cortex in the mixing ratios  
30 mentioned above may be used as composed, the composition would preferably be used in pharmaceutical preparations formulated by adding conventional pharmaceutically acceptable excipients to the composition. The pharmaceutical preparations used for this purpose include tablets, ointments, solutions (gargles), sprays, etc. For example, the composition  
35 of the present invention may be included in the toothpaste or formulated

as tablets for internal use, ointments for external use, or solutions by mixing the extracts with pharmaceutically acceptable carriers.

In addition, the composition of the present invention may be included in conventional gargle preparations for washing the mouth, or it may be mixed with a propellant to prepare a spray packaged in a pressurized container and then sprayed onto the attacked periodontal portion, or it may be included in toothpaste which is used over a long period of time to obtain the preventive and therapeutic effect on periodontal diseases.

The effective dosage of the present composition can be varied depending on the purpose of use, the severity of periodontal disease to be treated, the preparation employed, etc., but is generally from 50mg to 5g, particularly from 100mg to 3g, per day for an adult man, as the combination of the extracts of *Zizyphi fructus* and *Magnoliae cortex*.

The content of the combination of the extracts of *Zizyphi fructus* and *Magnoliae cortex* in the total composition may be varied depending on the purpose of its use. However, when the content of the active ingredients is too low, the pharmaceutical effect of the composition rapidly decreases, whereas when their content is too high, the quality of the product may be reduced due to the colouring effects of the extracts of *Zizyphi fructus* and *Magnoliae cortex*. Based on the effective dosages given above, it is preferable to formulate the unit dosage form so that a tablet contains from 50mg to 250mg of the combination of the present invention and the ointment, solution (gargle), toothpaste and spray contain the combination of the present invention in the concentration of 0.2 to 5.0 wt%.

30

When the extracts of *Zizyphi fructus* and *Magnoliae cortex* are orally administered to mice and rats, the LD<sub>50</sub> level of each extract, which was used as the standard of acute toxicity, was determined to be 5000mg/kg or more. It is therefore evident that they are safe medicinal substances.

35

To increase the desired pharmacological effect, the composition of the present invention can further contain additional medicinal herbs used to prevent and treat periodontal diseases, for example, extract of Ginkgo folium, Myrrha, extract of Mori radice cortex, Sanguinaria extract,  
5 extract of Zea mays, etc.

The present invention will be more specifically illustrated by the following examples and experiments. However, it should be noted that these examples are intended to help the understanding of the present  
10 invention and that the technical scope of the present invention is not limited by these examples in any manner.

**Example 1 : Preparation of the extract of Magnoliae cortex**

15 500g of Magnoliae cortex was sliced off and then extracted twice with 2.5 l of 70% ethanol for 3 hours, each time while refluxing in a water bath. The extracts were combined and then filtered. The filtrate was concentrated to obtain 60g of the extract of Magnoliae cortex.

20

**Example 2 : Preparation of the extract of Zizyphi fructus**

300g of Zizyphi fructus was extracted twice with 1.5 l of 70% ethanol for 3 hours, each time while refluxing in a water bath. The  
25 extracts were combined and then filtered. The filtrate was concentrated to obtain 140g of the extract of Zizyphi fructus.

The composition comprising the combination of the extracts of Zizyphi fructus and Magnoliae cortex can be prepared in tablet, ointment,  
30 toothpaste, solution (gargles) or spray form using conventional methods in the pharmaceutical field. In the preparation, the mixing ratio by weight of the extracts of Zizyphi fructus and Magnoliae cortex ranges from 1:6 to 1:12, particularly, and preferably from 1:8 to 1:10, in view of the following experimental results. The specific examples of the present  
35 composition are as follows.

**Composition 1 : Film coated tablet (in each 600mg tablet)**

	Extract of Zizyphi fructus	20mg
	Extract of Magnoliae cortex	200mg
5	Lactose	355mg
	Hydroxypropylcellulose	5mg
	Magnesium stearate	3mg
	Glycerine	2mg
	Hydroxypropylmethylcellulose	15mg

10

The tablet was prepared from the above mentioned components using conventional method for preparing tablets.

**Composition 2 : Ointment (in wt%)**

15

	Extract of Zizyphi fructus	0.04
	Extract of Magnoliae cortex	0.32
	Carboxyvinyl polymer	7.00
	Glycerol	30.00
20	Peppermint oil	0.006
	Propylene glycol	30.00
	Purified water to make	100.

25 The ointment was prepared from the above mentioned components using the conventional method for preparing ointments.

**Composition 3 : Toothpaste (in wt%)**

	Extract of Zizyphi fructus	0.04
30	Extract of Magnoliae cortex	0.40
	Alumina	45.00
	Propylene glycol	10.00
	Carboxymethylcellulose sodium	2.00
	Stevioside	0.30
35	Sodium lauryl sulfate	1.00

Peppermint	0.50
Purified water to make	100.

The above components were mixed using conventional method for  
5 preparing toothpastes.

**Composition 4 : Solution (in wt%)**

Extract of Zizyphi fructus	0.08
10 Extract of Magnoliae cortex	0.96
Polyethylene glycol	7.00
Ethanol	5.00
Peppermint oil	0.06
Purified water to make	100.

15

The solution was prepared by mixing and dissolving the above mentioned components and then adjusting the pH value to 5.0 using conventional method for preparing solutions.

20 **Composition 5 : Spray (in wt%)**

Extract of Zizyphi fructus	0.04
Extract of Magnoliae cortex	0.24
Trichloromonofluoromethane	40.00
25 Dichlorodifluoromethane	45.00
Peppermint oil	0.15
Ethanol	6.00
Polyethylene glycol	8.65

30 The spray was prepared by mixing the above mentioned components and then packaging the mixture into a pressurized container using conventional method for preparing sprays.

**Experiment : Determination of the mixing ratio of the extract f**  
35 **Zizyphi fructus and the extract of Magnoliae cortex**

**Experiment 1 : Determination of the activity of gingival fibroblast**

To determine the mixing ratio of the extract of Zizyphi fructus to the extract of Magnoliae cortex in the present composition that exhibits the optimum tissue regenerating effect, the effect of the combination of two extracts on the activity of gingival fibroblasts was measured by varying the mixing ratio of two extracts. For this experiment, gingival fibroblasts were obtained by collecting the gingiva of first premolar from the patients attending Seoul National University Hospital for teeth correction. Just before collecting the gingiva, dental calculus and plaque were removed using curette and the mouth was washed several times with 0.1% cyclohexidine digluconate solution. Then, the gingiva was locally anesthetized and then the normal gingival tissues were taken by incising the inner slanting surface between teeth. The collected tissue specimen was incubated in the  $\alpha$ -MEM medium (Gibco, Inc., Grand Island, N.Y., U.S.A.) supplemented with 100U/ml of penicillin, 100 $\mu$ g/ml of streptomycin and 10% FBS and then subjected to subculture 5 times while replacing the medium with the same volume of fresh medium at the intervals of three days. During incubation, humidity of 95% and temperature of 37°C were maintained and 95% air and 5% CO<sub>2</sub> were continuously supplied. The subcultured gingival fibroblast was treated with 0.25% trypsin-EDTA (ethylenediaminetetraacetate) solution and centrifuged at 1200rpm to obtain the cell suspension in the medium. This suspension was distributed into 96-well plate using standard hemocytometer in an amount of  $1 \times 10^5$  cells per well and then incubated at 37°C, 5% CO<sub>2</sub> for one day. After one day, each well was washed with HBSS (Hank's Balanced Salt Solution) and then the medium was replaced with fresh medium. As the experimental preparation for the positive control, platelet derived growth factor (PDGF-BB, Genzyme, Co., Cambridge, M.A., U.S.A.) was dissolved in distilled water as the solvent, and the extract of Magnoliae cortex was dissolved in ethanol (EtOH) and the extracts of Zizyphi fructus and Ginkgo folium were dissolved in dimethylsulfoxide (DMSO). Each of the experimental preparations was added to the culture solution at the concentration described in the following table and the incubation was continued for 24 hours while

maintaining humidity of 95% and temperature of 37°C and continuously supplying 95% air and 5% CO<sub>2</sub>. After completion of incubation, 50 $\mu$ l of the solution of MTT(methyl thiazol-2-yl-2,5-diphenyltetrazolium bromide, Sigma Co., St. Louis, M.O., U.S.A.) dissolved in physiological saline was added to each well and then the cell was incubated for further 4 hours under the same condition as above. Then, MTT solution was filtered off under reduced pressure and the formazon crystal thus produced was dissolved by adding 50 $\mu$ l of DMSO to each well. The plate was thoroughly shaken and then the optical density at 570nm was measured using ELISA reader (Thermo max, molecular devices, Menlo Park C.A., U.S.A.). In this experiment, the control group used only  $\alpha$ -MEM medium free from test substances. All the test results as measured were calculated as the percentage of the control group and are described in the following Table 1.

Table 1. Effect of the extract of Zizyphi fructus on the activity of gingival fibroblast

Extract of Zizyphi fructus	Cell activity (%)	Increase (%)
Negative control (no treatment)	100.0	0.0
Ethanol (0.5%)	95.8	-4.2
2 $\mu$ g/ml	103.1	3.1
6 $\mu$ g/ml	112.6	12.6
10 $\mu$ g/ml	118.5	18.5
14 $\mu$ g/ml	118.8	18.8

Note : Increase (%) =  $(T - C)/C \times 100$

In the above, T abbreviates the cell activity with test substance and C abbreviates the cell activity in the negative control group.



From the result described in the above Table 1, it can be seen that the extract of Zizyphi fructus increases the cell activity in proportion to the increase in its concentration and shows the maximum effect at the concentration of 10 $\mu$ g/ml or more.

5

Table 2. Effect of the extract of Magnoliae cortex on the activity of gingival fibroblast

10	Extract of Magnoliae cortex	Cell activity (%)	Increase (%)
	Negative control (no treatment)	100.0	0.0
	DMSO (0.5%)	94.2	-5.8
15	20 $\mu$ g/ml	99.8	-0.2
	60 $\mu$ g/ml	99.2	-0.8
	100 $\mu$ g/ml	98.7	-1.3
	140 $\mu$ g/ml	95.1	-4.9

20

Note : Increase (%) =  $(T - C)/C \times 100$

In the above, T abbreviates the cell activity with test substance and C abbreviates the cell activity in the negative control group.

25

From the result described in the above Table 2, it can be seen that the extract of Magnoliae cortex reduces cell activity as its concentration increases.

30

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Table 3. Effect of the combination of the extracts of Zizyphi fructus and Magnoliae cortex on the activity of gingival fibroblast

Composition	Mixing ratio	Cell activity (%)	Increase (%)
Negative control (no treatment)		100.0	0.0
DMSO(0.5%)		94.2	-5.8
Ethanol(0.5%)		95.8	-4.2
PDGF(1mg/ml)		120.1	20.1
EZF 10μg/ml		118.5	18.5
EMC 100mg/ml		98.7	-1.3
EZF 10μg/ml + EMC 10μg/ml	1:1	119.1	19.1
EZF 10μg/ml + EMC 20μg/ml	1:2	120.6	20.6
EZF 10μg/ml + EMC 40μg/ml	1:4	122.4	22.4
EZF 10μg/ml + EMC 60μg/ml	1:6	127.9	27.9
EZF 10μg/ml + EMC 80μg/ml	1:8	129.9	29.9
EZF 10μg/ml + EMC 100μg/ml	1:10	131.5	31.5
EZF 10μg/ml + EMC 120μg/ml	1:12	127.6	27.6
EZF 10μg/ml + EMC 140μg/ml	1:14	120.4	20.4
EZF 10μg/ml + EMC 160μg/ml	1:16	112.3	12.3
EZF 10μg/ml + EMC 180μg/ml	1:18	102.9	2.9
EGF 20μg/ml + EMC 120μg/ml	1:6	122.3	22.3

Note : EZF = Extract of Zizyphi fructus

EMC = Extract of Magnoliae cortex

EGF = Extract of Ginkgo folium

Increase (%) =  $(T - C)/C \times 100$

In the above, T abbreviates the cell activity with test substance and C abbreviates the cell activity in the negative control group.

As can be seen from the result described in the above Tables 1 to 3, when the extract of Zizyphi fructus and the extract of Magnoliae cortex is each used alone, the extract of Zizyphi fructus enhances the activity of gingival fibroblasts while the extract of Magnoliae cortex inhibits the activity of gingival fibroblasts. However, when the combination of the extract of Zizyphi fructus and the extract of Magnoliae cortex is used with the mixing ratios ranging from 1:6 to 1:12 on the basis of weight, effect on the activity of gingival fibroblasts is far better than that of the extract of Zizyphi fructus alone and, particularly, with mixing ratios ranging from 1:8 to 1:10 on the basis of weight the activity of gingival fibroblast has greatly increased. It is considered that the result that the combination of these two extracts increase activity of gingival fibroblast, is due to the interaction of these two extracts. In addition, it can be also identified that this increased activity is superior to the effect of PDGF alone and the effect of composition of extracts of Ginkgo folium and Magnoliae cortex, which are typically used to increase the activity of gingival fibroblast. Accordingly, it is identified that the composition of extracts of Zizyphi fructus and Magnoliae cortex according to the present invention shows a synergistic effect at certain mixing ratios in comparison to the use of each extract alone and is evidently a good agent for treatment of periodontal disease.

#### Experiment 2 : Determination of anti-inflammatory effect

The anti-inflammatory effect of the composition containing the extracts of Zizyphi fructus and Magnoliae cortex according to the present invention was determined by measuring the effect of inhibiting PGE<sub>2</sub> production of gingival fibroblast in vitro. The gingival fibroblast subcultured 5 to 7 times was distributed in 24-well plate in the concentration of 10<sup>5</sup> cells in 1ml of  $\alpha$ -MEM medium (Gibco, Inc. Grand Island, N.Y., U.S.A.) containing 1% antibiotics and 10% FBS per each well, and then 1ng/ml of rHUIL-1 $\beta$  (Genzyme, Co., Cambridge, M.A., U.S.A.) was added to each well to induce the production of PGE<sub>2</sub>. To the culture solution in each well was added each of the extract of

Magnoliae cortex dissolved in ethanol (EtOH) and the extract of Zizyphi fructus and the extract of Ginkgo folium dissolved in dimethylsulfoxide, respectively, at the concentration as described in the following table and the well was aseptically incubated at 37°C under 5% CO<sub>2</sub> for 48 hours with using the well free from any test substances as the control group. After completion of incubation, the medium of each well was collected and PGE<sub>2</sub> in the medium was analyzed according to colorimetry at 450nm using ELISA reader in PGE<sub>2</sub> enzyme immunoassay system (Amersham, In. Buckinghamshire, U.K.). The result as measured is described in the following Tables 4 to 6.

Table 4. Effect of the extract of Zizyphi fructus on the inhibition of PGE<sub>2</sub> production

Extract of Zizyphi fructus	PGE <sub>2</sub> production (Pg)	Inhibition (%)
Negative control (no treatment)	15.7	-
IL-1 $\beta$ (positive control)	54.3	0.0
IL-1 $\beta$ + 2 $\mu$ g/ml	32.4	40.3
IL-1 $\beta$ + 6 $\mu$ g/ml	25.4	53.2
IL-1 $\beta$ + 10 $\mu$ g/ml	20.8	61.7
IL-1 $\beta$ + 14 $\mu$ g/ml	19.2	64.6

Note : Inhibition (%) = (PC - TC)/PC  $\times$  100

In the above, PC abbreviates the PGE<sub>2</sub> production in positive control group and TC abbreviates the PGE<sub>2</sub> production with test substances.

From the result described in the above Table 4, it can be seen that the extract of Zizyphi fructus inhibits PGE<sub>2</sub> production in proportion

to the increase in its concentration and shows the maximum effect at the concentration of 10 $\mu$ g/ml or more.

Table 5. Effect of the extract of Magnoliae cortex on the inhibition of PGE<sub>2</sub> production

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Extract of Magnoliae cortex	PGE <sub>2</sub> production (Pg)	Inhibition (%)
Negative control (no treatment)	15.7	-
IL-1 $\beta$ (positive control)	54.3	0.0
IL-1 $\beta$ + 20 $\mu$ g/ml	26.4	51.4
IL-1 $\beta$ + 60 $\mu$ g/ml	18.1	66.7
IL-1 $\beta$ + 100 $\mu$ g/ml	14.9	72.6
IL-1 $\beta$ + 140 $\mu$ g/ml	14.1	74.0

Note : Inhibition (%) = (PC - TC)/PC  $\times$  100

20

In the above, PC abbreviates the PGE<sub>2</sub> production in positive control group and TC abbreviates the PGE<sub>2</sub> production with test substances.

25

From the result described in the above Table 5, it can be seen that the extract of Magnoliae cortex inhibits PGE<sub>2</sub> production in proportion to the increase in its concentration and shows the maximum effect at concentrations of 100 $\mu$ g/ml or more.

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Table 6. Effect of the combination of the extracts of Zizyphi fructus and Magnoliae cortex on the inhibition of PGE<sub>2</sub> production

Composition	Mixing ratio	PGE <sub>2</sub> production (Pg)	Inhibition (%)
Negative control (no treatment)		15.7	-
IL-1 $\beta$ (positive control)		54.3	0.0
IL-1 $\beta$ +EZF 10 $\mu$ g/ml		20.8	61.7
IL-1 $\beta$ +EMC 100 $\mu$ g/ml		14.9	72.6
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 10 $\mu$ g/ml	1:1	13.6	75.0
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 20 $\mu$ g/ml	1:2	10.9	79.9
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 40 $\mu$ g/ml	1:4	8.4	84.5
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 60 $\mu$ g/ml	1:6	6.7	87.7
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 80 $\mu$ g/ml	1:8	6.1	88.8
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 100 $\mu$ g/ml	1:10	5.2	90.4
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 120 $\mu$ g/ml	1:12	6.4	88.2
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 140 $\mu$ g/ml	1:14	8.8	83.8
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 160 $\mu$ g/ml	1:16	10.6	80.5
IL-1 $\beta$ +EZF 10 $\mu$ g/ml+EMC 180 $\mu$ g/ml	1:18	14.4	73.5
IL-1 $\beta$ +EGF 20 $\mu$ g/ml+EMC120 $\mu$ g/ml	1:6	15.8	70.9

Note : EZF = Extract of Zizyphi fructus

EMC = Extract of Magnoliae cortex

EGF = Extract of Ginkgo folium

Inhibition (%) = (PC - TC)/PC  $\times$  100

In the above, PC abbreviates the PGE<sub>2</sub> production in the positive control group and TC abbreviates the PGE<sub>2</sub> production with test substances.

As can be seen from the result described in the above Tables 4 to

6, when the extract of Zizyphi fructus and the extract of Magnoliae cortex is each used alone, the extract of Zizyphi fructus slightly inhibits the production of PGE<sub>2</sub> which mediates the inflammatory reaction and the extract of Magnoliae cortex considerably inhibits PGE<sub>2</sub> production. However, when the combination of the extract of Zizyphi fructus and the extract of Magnoliae cortex is used in mixing ratios ranging from 1:6 to 1:12 on the basis of weight, their effect on the inhibition of PGE<sub>2</sub> production are far better than those of the extract of Magnoliae cortex and at other mixing ratio the effect of inhibiting the PGE<sub>2</sub> production is reduced. This observation resulted from the fact that by using the combination of the extract of Zizyphi fructus and the extract of Magnoliae cortex the extract of Zizyphi fructus potentiates the effect of inhibiting PGE<sub>2</sub> production of the extract of Magnoliae cortex. In addition, it can be also identified that such increased effect is superior to the effect of the composition of extracts of Ginkgo folium and Magnoliae cortex as the typical substances known as having the best effect for treating periodontal diseases. Accordingly, it is identified that the composition of extracts of Zizyphi fructus and Magnoliae cortex according to the present invention shows a synergistic anti-inflammatory effect on periodontal diseases at the certain mixing ratio in comparison to the single use of each extract and is evidently a good agent for treatment of periodontal diseases.

### Experiment 3 : Determination of antibacterial effect

To determine the antibacterial effect of the composition of the extracts of Zizyphi fructus and Magnoliae cortex according to the present invention, the following test was conducted using anerobic gram-negative strain, *Porphyromonas gingivalis* W50 which is distributed by Dental College, University of New York State in U.S.A., *Prevotella intermedia* ATCC25611 and aerobic gram-negative strain, *Actinobacillus actinomyces-temcomitans* Y4.

*Porphyromonas gingivalis* W50 (PG) and *Prevotella intermedia* ATCC 25611 (PI) were inoculated onto trypticase soy agar blood medium

containing 5 $\mu$ g of hemin and 0.5 $\mu$ g of menadione and then incubated in the manner of pure culture in an anerobic incubator containing 10% CO<sub>2</sub>, 10% H<sub>2</sub> and 80% N<sub>2</sub> at 37°C. *Actinobacillus actinomycetemcomitans* Y4 (AA) was inoculated onto trypticase soy agar blood medium and then

5 aerobically incubated in the manner of pure culture in a bacterial incubator containing 10% CO<sub>2</sub>. Each bacterial strain was purely incubated for 4 to 7 days, then inoculated onto trypticase soy broth and incubated for 48 to 72 hours in either an anerobic or an aerobic incubator as mentioned above until the bacterial count reaches 10<sup>7</sup> cells/ml. The

10 incubated bacterial strains were inoculated onto the blood agar medium combined with the test substances in the concentration as described in the following table using a multi-inoculator. The medium was incubated in an anerobic incubator containing 10% CO<sub>2</sub>, 10% H<sub>2</sub> and 80% N<sub>2</sub> at 37 °C for 3 to 5 days and the growth of bacterial strains was then visually

15 identified to determine the minimum inhibitory concentration (MIC) of the test substance on the growth of each strain. The measured MICs are described in the following Table 7.

Table 7. MICs on causative organisms of periodontal diseases

20

(unit :  $\mu$ g/ml)

Composition (Mixing ratio)	Strains		
	PG	PI	AA
25 EZF	2560	2560	2560
EMC	160	160	160
EZF + EMC (1:4)	40	80	80
EZF + EMC (1:8)	40	40	80
30 EZF + EMC (1:10)	40	80	80
EZF + EMC (1:12)	80	80	80
EGF + EMC (1:6)	160	160	320

35 Note : EZF = Extract of Zizyphi fructus



EMC = Extract of Magnoliae cortex

EGF = Extract of Ginkgo folium

As can be seen from the result described in the above Table 7,  
5 the composition of the extracts of Zizyphi fructus and Magnoliae cortex,  
which was identified as showing a synergistic effect in the experiments  
for gingival fibroblast activity in Experiment 1 and for showing an anti-  
inflammatory effect in Experiment 2, also exhibits a superior antibacterial  
effect at a mixing ratio of the two extracts ranging from 1:6 to 1:12 on  
10 the basis of weight, in comparison to the single use of each extract.  
This observation resulted from the fact that although the extract of  
Zizyphi fructus itself has substantially no antibacterial effect, its  
combination with the extract of Magnoliae cortex increases the  
antibacterial effect of the extract of Magnoliae cortex. In addition, it  
15 was also shown that such increased effect is superior to the effect of the  
composition of extracts of Ginkgo folium and Magnoliae cortex as the  
typical substances known as having the best effect for treating  
periodontal diseases. Accordingly, it is identified that the composition of  
extracts of Zizyphi fructus and Magnoliae cortex according to the present  
20 invention shows an evident synergistic antibacterial effect in comparison  
to the single use of each extract.

#### **Experiment 4 : Effect on bone regeneration**

25 To determine the tissue regenerating effect of the composition  
containing the extract of Zizyphi fructus and the extract of Magnoliae  
cortex according to the present invention, the following experiment was  
conducted using calvaria of mouse and unsaponified extract of Zea mays  
as the positive control.

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In this experiment, as the composition of the present invention the  
combination of the extract of Zizyphi fructus and the extract of  
Magnoliae cortex in the mixing ratio ranging from 1:10 on the basis of  
weight was administered daily in an amount of 0.1g (first group) or 0.5g  
35 (second group) per kg of mouse body weight, and as the positive control

group the unsaponified extract of Zea may was also administered daily in an amount of 0.1g (third group) or 0.5g (fourth group) per kg of mouse body weight. Mouse not receiving any test substance was used as the negative control group (fifth group). The daily dosage of each test substance was divided into two, each of which was dissolved in 1ml of distilled water and then administered directly to mouse stomach via oral zonde needle in the morning and in the afternoon, respectively.

Mouse weighing 200-250g was anesthetized with 30mg/kg of pentobarbital sodium (Hanlim Pharm. Co., Seoul, Korea) by intraperitoneal injection. Then, hair of mouse head was cut and then the portion to be operated was disinfected with 0.5% chlorhexidine. For the rest and convenience during operation, mouse head was fixed with cephalostat, and then cut out along the centerline from the front part of frontal bone to the rear part of occipital bone. The skin and mucous membrane were pulled outside to expose the calvaria. One side of temporal bone was perforated in a diameter of 5mm using trephine bur (3i, Florida, U.S.A.) without any defect to meninges and then sutured. Each of the test substances was administered in the manner as mentioned above and, after 2 or 3 weeks mouse was sacrificed to remove the calvaria which was fixed in 10% formalin, introduced into 5% trichloroacetic acid for 5 days to remove the gray matter, and then subjected to dehydration and paraffin embedding. The embedded specimen was sliced into sections having thickness of 6 $\mu$ m and dyed with Masson-trichrome. Then the tissues were observed using an optical microscope, Olymplus BH-2 (Olymplus Optical Co., Ltd., Osaka, Japan). The results as observed are shown in Figures 1 to 10.

From Figures 1 to 10, it can be seen that in the calvaria of mouse of first and second groups receiving the present composition, after 2 weeks from administration of the composition the new bone and bone-like tissues were actively produced and after 3 weeks the osteogenesis was progressed to substantially complete the unification. This result is far better than that obtained in the third and fourth groups as the positive control groups receiving the unsaponified extract of Zea mays. Contrary

to this, in the fifth group as the negative control group not receiving any test substance, it can be found that the defected portion of calvaria was filled with fibrous connective tissues. From this result, it can be identified that the present composition containing the combination of  
5 extract of Zizyphi fructus and extract of Magnoliae cortex shows a good tissue regenerating effect to actively regenerate the bone tissue such as alveolar bone defected in periodontal diseases.

As can be demonstrated by the above experiments, the  
10 composition of the present invention comprising the extract of Zizyphi fructus and the extract of Magnoliae cortex in the mixing ratio ranging from 1:6 to 1:12 on the basis of weight can overcome the one-sided effects obtained from the single use of each extract to provide various therapeutic effects on periodontal diseases. The effect of the present  
15 composition shows synergistically and is far better than that obtained from the single use of each extract.

#### Experiment 5 : Stability test

20 To determine the stability of the preparations produced in Compositions 1 to 5 above, each preparation was stored under the conditions described below and then the contents of magnolol in the extract of Magnoliae cortex contained in the preparation was analysed with HPLC using UV detector at 254nm. The result is described in the  
25 following Table 8.

#### Condition for storage :

- Test substances : The test samples prepared in Compositions 1 to 5  
30 were filled in a glass bottle or a compressed container, which was then plugged and put in a cardboard box.
- Temperature for storage : 40°C ( $\pm 1^\circ\text{C}$ )  
35 Provided that, the spray preparation of Composition 5 was stored at room temperature.

- Time for measurement : just after and 2 months, 4 months, 6 months after preparation
- Test item :
  - 5      Composition 1 (Film-coated tablet) : appearance, disintegration (artificial gastric juice), content
  - Composition 2 (Ointment) : appearance, content
  - Composition 3 (Toothpaste) : appearance, pH (10% suspension), content
  - 10     Composition 4 (Solution) : appearance, pH, content
  - Composition 5 (Spray) : content

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Table 8. Results of stability test

Test sample	Test item	Results			
		Just after	2 months	4 months	6 months
Composition 1	appearance	yellowish -brown coated tablet	yellowish -brown coated tablet	yellowish -brown coated tablet	yellowish -brown coated tablet
	disintegration (artificial gastric juice)	12 min.	14 min.	11 min.	12 min.
	content (magnolol)	100.3%	101.7%	98.7%	99.5%
Composition 2	appearance	pale yellow ointment	pale yellow ointment	pale yellow ointment	pale yellow ointment
	content (magnolol)	100.7%	100.5%	101.0%	99.7%
Composition 3	appearance	pale yellow paste	pale yellow paste	pale yellow paste	pale yellow paste
	pH	6.5	6.3	6.3	6.4
	content (magnolol)	104.0%	105.7%	103.2%	100.8%
Composition 4	appearance	pale yellow solution	pale yellow solution	pale yellow solution	brown solution
	pH	5.3	5.1	5.0	4.8
	content (magnolol)	101.4%	99.7%	99.0%	98.4%
Composition 5	content (magnolol)	101.6%	99.7%	99.5%	100.9%

As can be seen from the result described in above Table 8, after 6 months at 40°C the appearance of tablet, ointment, toothpaste, solution and spray preparations was substantially not changed and the content of

magnolol in the extract of Magnoliae cortex is 95% or more in comparison to that just after the preparation. Therefore, the preparations of the present invention are stable and have no difficulty in industrialization.

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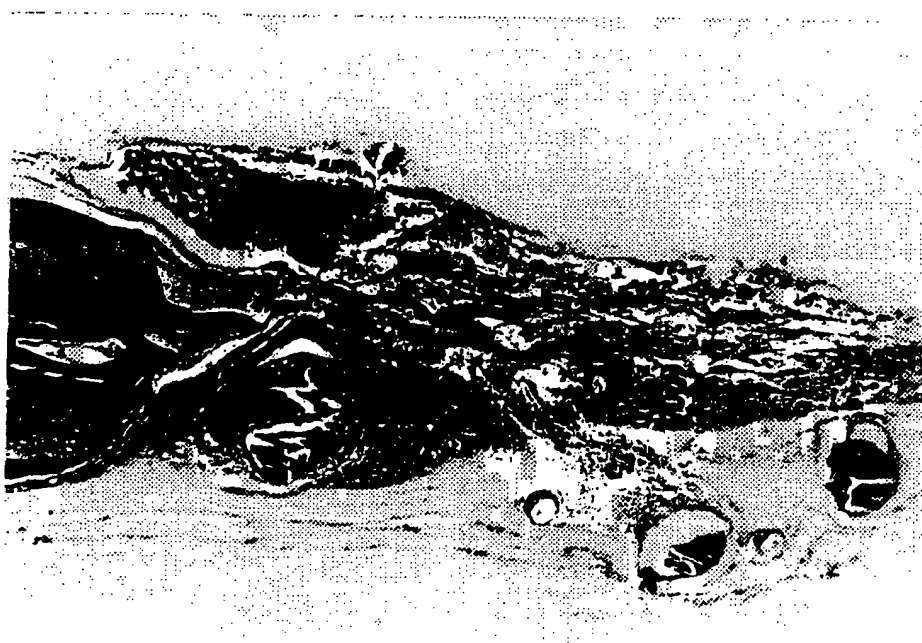
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**WHAT IS CLAIMED IS :**

1. A composition for the prevention and treatment of periodontal diseases which comprises an alcohol extract of Zizyphi fructus and an alcohol extract of Magnoliae cortex wherein the ratio of the weight of the extract of Zizyphi fructus to the weight of the extract of Magnoliae cortex ranges from 1:6 to 1:12.
2. The composition as defined in claim 1, wherein the ratio of the weight of the alcohol extract of Zizyphi fructus and the weight of the alcohol extract of Magnoliae cortex ranges from 1:8 to 1:10.
3. The composition as defined in claim 1 or 2, wherein the alcohol extract of Zizyphi fructus and the alcohol extract of Magnoliae cortex are ethanol extracts thereof.
4. The composition as defined in claim 1, which is formulated in a pharmaceutical unit dosage form using an additional pharmaceutically acceptable carrier.
5. The composition as defined in claim 4, wherein the pharmaceutical unit dosage form is tablet, ointment, toothpaste, solution (gargle) or spray.
6. The composition as defined in claim 5, which contains from 50mg to 250mg of the combination of the extract of Zizyphi fructus and the extract of Magnoliae cortex per tablet.
7. The composition as defined in claim 5, which contains the combination of the extract of Zizyphi fructus and the extract of Magnoliae cortex in the concentration of 0.2 to 5.0 wt% per unit dosage form of ointment, toothpaste, solution (gargle) or spray.

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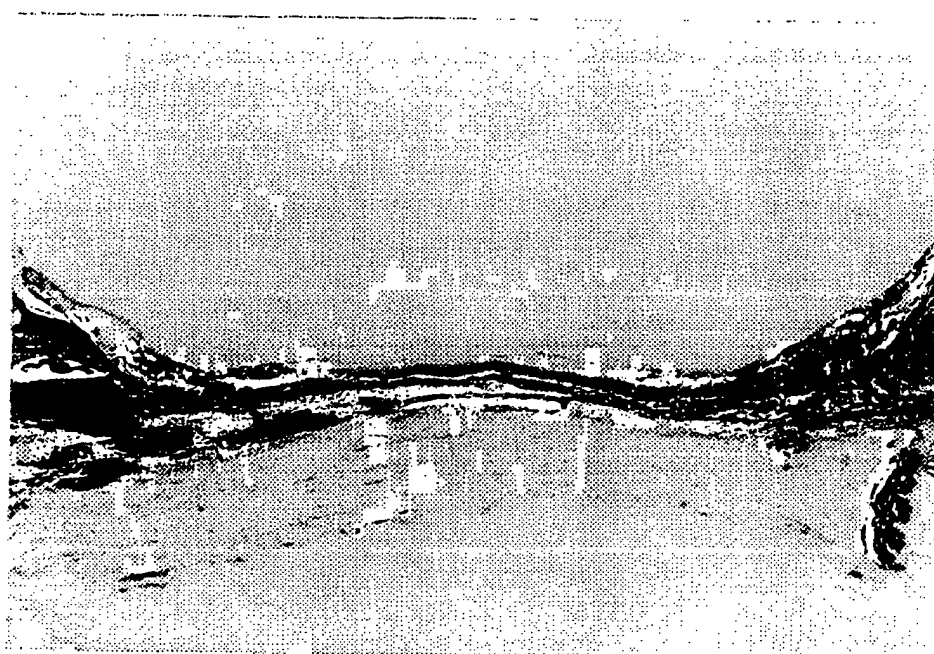
**Figure 1**





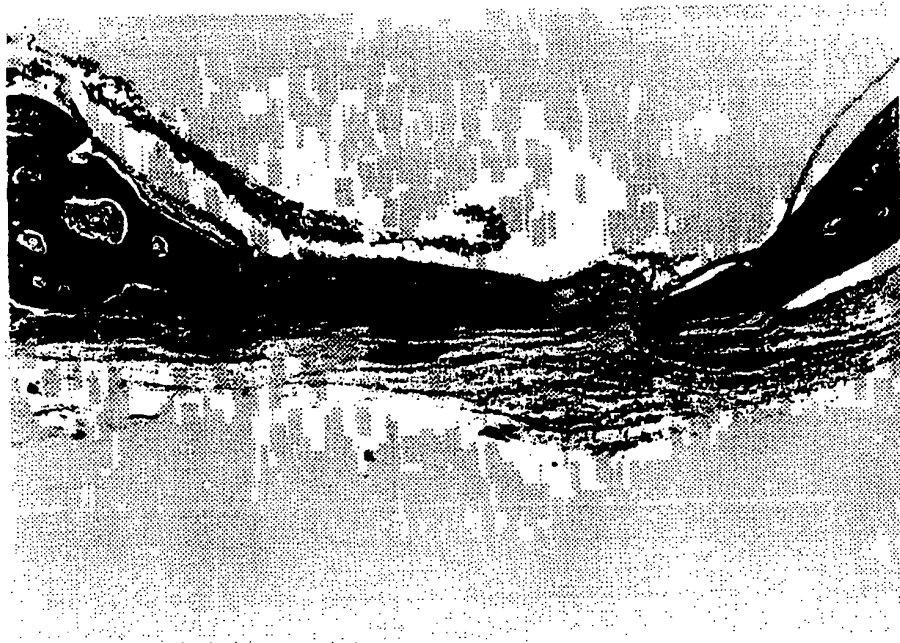
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**Figure 2**



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Figure 3



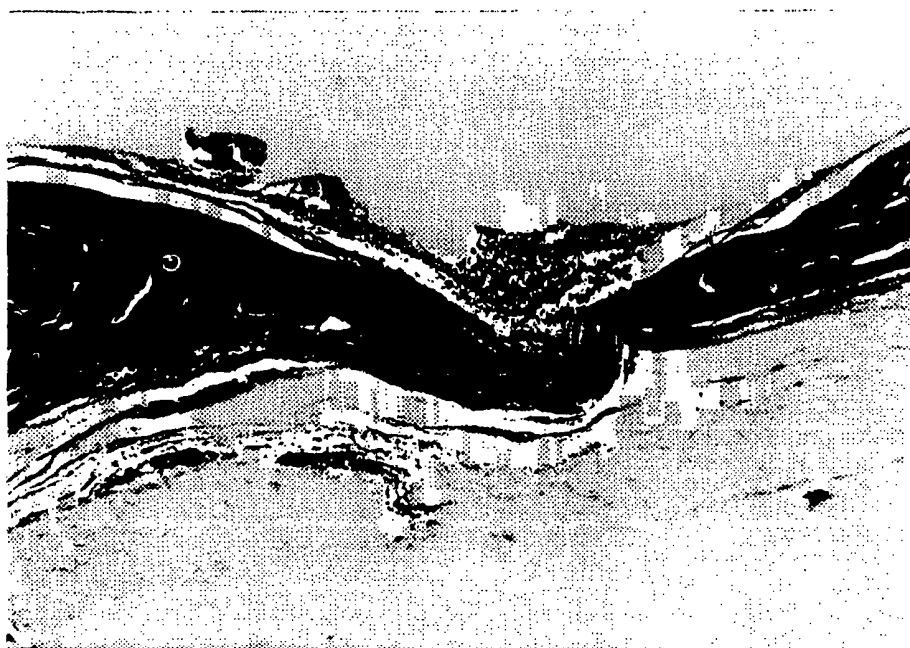
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Figure 4



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**Figure 5**



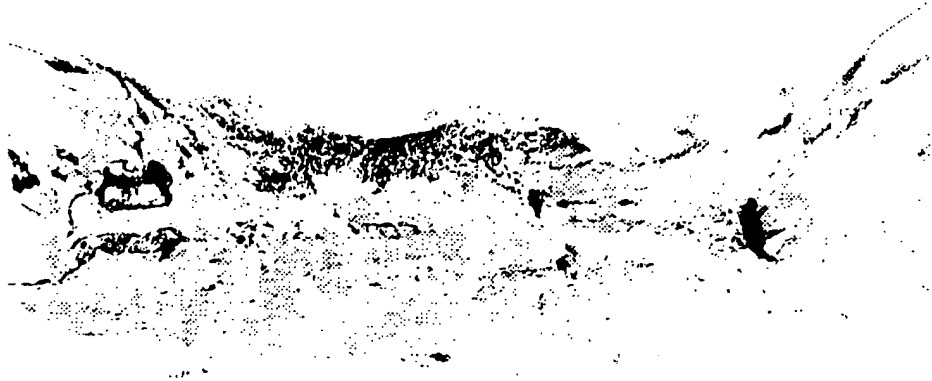
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**Figure 6**



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**Figure 7**



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**Figure 8**



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**Figure 9**





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**Figure 10**



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR 97/00048

## A. CLASSIFICATION OF SUBJECT MATTER

IPC<sup>6</sup>: A 61 K 35/78, A 61 K 7/26

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC<sup>6</sup>: A 61 K 35/78, A 61 K 7/26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPIL

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Database WPIL on Questel, London, Derwent Publications Ltd., AN 95-011734, JP 6-298633 A (CHUGAI PHARM CO LTD), abstract.  -----	1

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

\* Special categories of cited documents:

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05 June 1997 (05.06.97)

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